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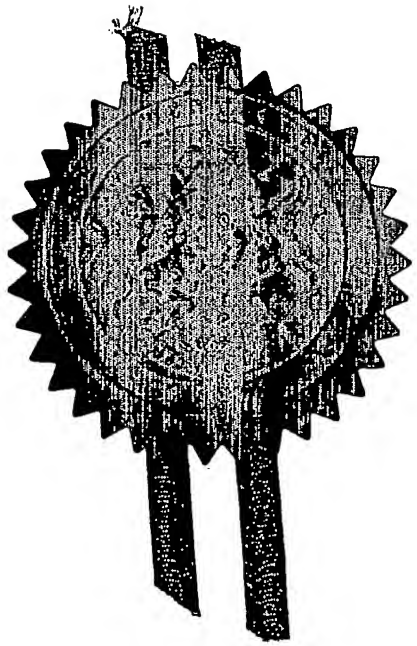

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"Insulin Releasing Peptides from Skin Secretions of Amphibians"

The present invention relates to the discovery of novel insulin-releasing peptides in the skin secretions of amphibians and their potential use as stimulators of pancreatic beta cell function in the treatment of diabetes mellitus.

Amphibian skin contains two kinds of secretory glands. Mucous glands are distributed throughout the body, and their secretions mainly provide the moist coating necessary for cutaneous respiration. The granular glands, also known as serous or poison glands may be distributed or concentrated in certain areas of the body. The secretions from the granular glands of anurans (frogs and toads) have been shown to contain pharmacologically active substances ranging from simple amines such as norepinephrine and histamine to biologically active peptides, piperidine and steroidal alkaloids, bufodienolides and tetrodotoxin [1-2]. These compounds are thought to play various roles, either in the regulation of physiological functions of the skin or in defence against predators or microorganisms [3-4].

During evolution some of these agents, or closely related daughter molecules, may have taken on distinct physiological roles in humans. It follows that pharmacologically active substances isolated from the skin secretions of frogs and toads may be useful for the treatment of human clinical disease such as diabetes mellitus.

The invention will now be demonstrated with reference to the following non-limiting examples and the accompanying figures wherein:

Figure 1 illustrates that semi-preparative C18 HPLC fractions of the skin secretions of *Agalychnis calcarifer* stimulate insulin secretion from BRIN-BD11 cells.

Figure 2 illustrates that semi-preparative C18 HPLC fractions of the skin secretions of *Agalychnis litodryas* stimulate insulin secretion from BRIN-BD11 cells.

Figure 3 illustrates that semi-preparative C18 HPLC fractions of the skin secretions of *Bombina variegata* stimulate insulin secretion from BRIN-BD11 cells.

Figure 4 illustrates that semi-preparative C18 HPLC fractions of the skin secretions of *Phyllomedusa trinitatis* stimulate insulin secretion from BRIN-BD11 cells.

Figure 5 illustrates that semi-preparative C18 HPLC fractions of the skin secretions of *Rana palustris* stimulate insulin secretion from BRIN-BD11 cells.

Figure 6 illustrates that semi-preparative C18 HPLC fractions of the skin secretions of *Rana pipiens* stimulate insulin secretion from BRIN-BD11 cells.

Figure 7 illustrates that semi-preparative C18 HPLC fractions of the skin secretions of *Rana saharica* stimulate insulin secretion from BRIN-BD11 cells.

Figure 8 illustrates the dependence of the stimulatory effects of 1653.2 Da purified peptide (peak 1.10) from *Agalychnis calcarifer* on intracellular pathways mediated by protein kinase A (forskolin), protein kinase C (PMA) or pertussis toxin-sensitive G-proteins.

Figure 9 illustrates the dependence of the stimulatory effects of 1641.7 Da, 1662.6 Da, 1619.8 Da and 1650.5 Da purified peptides (peaks 21, 22, 23 and 24) from *Bombina variegata* on intracellular pathways mediated by protein kinase A (forskolin), protein kinase C (PMA) or pertussis toxin-sensitive G-proteins.

Figure 10 illustrates the dependence of the stimulatory effects of 4920.4 Da and 4801.2 Da purified peptides (peaks 5.1 and 5.4) from *Rana saharica* on intracellular pathways mediated by protein kinase A (forskolin), protein kinase C (PMA) or pertussis toxin-sensitive G-proteins.

EXAMPLE 1

Collection of skin secretions: Captive bred of *Agalychnis calcarifer, Agalychnis litodryas, Bombina variegata, Phyllomedusa trinitatis, Rana palustris, Rana pipiens* and *Rana saharica* were maintained *in terraria* at 24 °C with 12 h/12 h light/dark cycle and fed on crickets. The skin secretions were obtained from groups of four amphibians from each species by gentle electrical stimulation (4-ms pulse width, 50 Hz, 5 V) using platinum electrodes rubbed over the moistened dorsal skin surface for 10s. Secretions were washed off into a glass beaker, using deionised water. The resultant secretions were freeze dried in a Hetosicc 2.5 freeze dryer (Heto, UK). Approximately 50 mg, dry weight, of skin secretion was obtained for each species.

Purification of peptide: Lyophilized crude venom (20mg) from each species was dissolved in 0.12% trifluoroacetic acid/water (2ml) and 1 ml of it was

chromatographed on a Vydac 218TP510 semi-preparative C-18 column (25 x 1cm, Hesperia, California, USA). The column was equilibrated with 0.12% (v/v) trifluoroacetic acid/water at a flow rate of 2ml/min. Using 0.1% (v/v) TFA in 70% acetonitrile/water, concentration of acetonitrile in the eluting solvent was raised to 80% (v/v) over 80 min using linear gradients. Absorbance was monitored at 214nm with collection of 2ml fractions. Fractions which showed major insulin releasing activity were pooled and rechromatographed using a Vydac 208TP54 analytical C-18 column (25 x 0.46 cm). The column was equilibrated with 0.12% (v/v) trifluoroacetic acid/water at a flow rate of 1ml/min. Using 0.1% (v/v) TFA in 70% acetonitrile/water, the concentration of acetonitrile in the eluting solvent was raised to 15% (v/v) over 5 min and to 80% (v/v) over 70 min using linear gradients. Absorbance was monitored at 214nm.

Culture of insulin-secreting cells: Clonal rat insulin-secreting BRIN-BD11 cells were cultured in RPMI-1640 tissue culture medium containing 10% (v/v) foetal calf serum, 1% (v/v) antibiotics (100 U/ml penicillin, 0.1 mg/ml streptomycin) and 11.1 mM glucose. The production and characterisation of BRIN-BD11 cells are described elsewhere [5]. Cells were maintained in sterile tissue culture flasks (Corning, Glass Works, UK) at 37 °C in an atmosphere of 5% CO_2 and 95% air using LEEC incubator (Laboratory Technical Engineering, Nottingham, UK). In three experimental series using purified peptides from *Agalychnis calcarifer*, *Bombina variegata* and *Rana saharica* cells were cultured overnight with 25 µM forskolin, 10 nM PMA or 0.1 µg/ml pertussis toxin prior to acute tests.

Acute insulin release studies: Insulin release from BRIN-BD11 cells was determined using cell monolayers [5]. The cells were harvested with the aid of trypsin/EDTA (Gibco), seeded into 24-multiwell plates (Nunc, Roskilde, Denmark) at a density of 1.5×10^6 cells per well, and allowed to attach overnight. Prior to acute test, cells were preincubated for 40 min at 37 °C in a 1.0 ml Krebs Ringer bicarbonate buffer (115 mM NaCl, 4.7 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM $NaHCO_3$, 5 g/l bovine serum albumin, pH 7.4) supplemented with 1.1 mM glucose. Test incubations were performed for 20 min at 37 °C using the same buffer supplemented with 5.6 mM glucose in the absence (control) and presence of various venom fractions, peaks (equivalent to approx. 25µl dried HPLC fraction) or test agents as indicated in the Figures. Cell viability after 20 min test incubations was assessed by modified neutral red assay [6]. After incubation, aliquots of buffer were removed and stored at -20 °C for insulin radioimmunoassay [7].

Molecular mass determination: The molecular masses of the purified individual non-toxic peaks exhibiting insulin releasing activity were determined using Matrix Assisted Laser Desorption Ionisation-Time of Flight (MALDI-TOF) mass spectrometry. Electrospray Ionisation quadripole ion-trap Mass Spectrometry (ESI-MS) was used for *Agalychnis calcarifer*, *Bombina variegata*, *Rana pipiens* and *Rana saharica*. Masses were recorded and compared with theoretical values calculated by the peptide calculator, a computer software package.

Depyroglutamation: Where necessary (*Bombina variegata*), pyroglutamate at the N-terminal was removed by adding 25 µl of pyroglutamate aminopeptidase preparation (50mM Na_2HPO_4, 10mM β-mercaptoethanol, 1 mM dithiothreitol, and 1mM EDTA

adjusted to pH 7.3 with H_3HPO_4) containing 0.4 mg/ml pyroglutamate aminopeptidase to 100µl of the lypophilised peptide. The reaction mix was incubated for 2 hours at 37 °C and then stored at –20 °C for subsequent amino acid determination by Edman degradation.

Structural analysis by automated Edman degradation: The primary structures of the purified peptides were determined by automated Edman degradation, using an Applied Biosystems Procisc 491 microsequencer. Standard operating proccdures were used (Applied Biosystems Model 491 Protein Sequencers Users Manual). The limit for detection of phenylthiohydantoin amino acids was 0.2 pmol. The primary structures were compared with those deposited in the SWISSPROT™ database.

Statistical analysis: Results are expressed as mean ± S.E.M. Values were compared using Student's unpaired t-test. Groups of data were considered to be significantly different if $P<0.05$.

Results

Isolation, mass spectrometry and sequence analysis of insulin-releasing releasing peptides: Skin secretions from the various amphibian species were purified by HPLC, yielding in each case multiple fractions that were subsequently screened for in vitro biological activity using BRIN-BD11 cells. The insulin-releasing profiles of peaks emerging from the primary HPLC separation are illustrated for *Agalychnis calcarifer*, *Agalychnis litodryas*, *Bombina variegata*, *Phyllomedusa trinitatis*, *Rana palustris*, *Rana pipiens* and *Rana saharica* in Figs. 1-7, respectively.

The major peaks of insulin-releasing activity were subjected to further HPLC purification steps, giving rise ultimately to the isolation of pure peptides with proven insulinotropic activity (Tables 1-7). Where sufficient sample was available, molecular masses and either partial or complete sequences were determined for each peptide as summarised for the various amphibian species in Tables 1-7. In instances where a complete sequence was obtained, the theoretical (calculated) molecular masses of the peptides were shown to corresponded closely to the experimental masses. This indicates the absence of any post-translational modification of constituent amino acids, such as phosphorylation, sulphation or glycation.

Six of the insulinotropic peptides have proved to be established structures. Peak 4.1 from *Rana pipiens* was identical to pipinin-1, peak 23 from *Bombina variegata* to bombesin, peak 2.10 from *Phyllomedusa trinitatis* to dermaseptin BIV precursor and peaks 5.4, 6.5 and 8.3 from *Rana saharica* matched Esculentin-1B, Brevinin-2EC and Brevinin-1E respectively. With these few exceptions, all other insulin-releasing peptides were novel structures as established using the SWISSPROT™ database. Even the functional observations with pipinin-1, dermaseptin BIV precursor, esculentin-1B and brevinin-2EC and 1E were novel as these were totally unsuspected insulin releasing peptides.

Modest similarity existed between some of the isolated insulin-releasing peptides and amphibian antimicrobial peptides that are unsuspected insulin secretagogues such as brevinin, dermaseptin, Rugosin A and tryptophyllin. However, unlike the latter agents, no evidence was obtained of cell lysis or a toxic action that might account for

insulin secretion. Thus the peptides reported herein appear to act through physiological mammalian processes controlling exocytosis of insulin.

To support this view, further studies were carried out using purified peptides from *Agalychins calcarifer, Bombina variegata* and *Rana saharica* to examine cellular mechanisms underlying the stimulation of insulin secretion. The stimulatory effects of the 1653.2 Da peptide (peak 1.10) from Agalychnis *calcarifer* (Figure 8) and 1641.7, 1662.6, 1619.8 and 1650.5 Da peptides (peaks 21, 22, 23 and 24 respectively) from *Bombina variegata* (Figure 9) were abolished in cells cultured overnight with forskolin to desensitise the cyclic AMP-protein kinase A pathway. Overnight culture with PMA or pertussis toxin did not affect the insulin-releasing ability of the peptides, suggesting lack of involvement of protein kinase C or G-protein dependent pathways. Overnight culture with forskolin or PMA resulted in the abolition of the acute stimulatory effects of forskolin or PMA, respectively (Figures 8 and 9). Interestingly, the insulin-releasing action of the 1653.2 Da peptide from *Agalychnis calcarifer* and peaks 21, 22, 23 and 24 from *Bombina variegata* were not affected by 50 μM verapamil and were clearly evident in cells depolarised with 30 mM KCl (Tables 8 and 9).

The stimulatory effects of the 4920.4 and 4801.2 Da peptides (peaks 5.1 and 5.4) from *Rana saharica* were abolished in cells cultured overnight with forskolin, PMA or pertussis toxin (Figure 10) indicating the involvement of both protein kinase A and C and pertussis toxin-sensitive G-protein in their stimulatory actions. As shown in Table 10, the insulin releasing actions of the isolated peptides were not inhibited by

the calcium channel blocker verapamil. Stimulatory effects on insulin secretion were also clearly evident in cells depolarised by 30 mM KCl.

Discussion

This research describes for the first time the isolation and characterisation of peptides with insulin-releasing activity from the skin secretions of *Agalychnis calcarifer, Agalychnis litodryas, Bombina variegata, Phyllomedusa trinitatis, Rana palustris, Rana pipiens* and *Rana saharica*. It is notable that this work has not only uncovered a diverse range of novel peptides structures but it has also revealed that the skin secretions from each amphibian species studied represents an unsuspected and rich source of peptides capable of stimulating physiological insulin secretion from mammalian pancreatic beta cells.

The insulin output induced by amphibian peptides is approximately equivalent to that induced by established mammalian gut peptides, GLP-1, GIP or CCK-8 [8-10]. This indicates that the amphibian peptides isolated are at least as capable as physiological mammalian hormones in stimulating insulin secretion. It is also clear that these peptides may also trigger insulin secretion and have other beneficial actions on beta cells which involve novel secretory pathways as suggested by studies using peak 1.10 from *Agalychnis calcarifer* and peaks 21, 22, 23 and 24 from *Bombina variegata*. In these cases the secretagogues appeared to be mediated through both protein kinase A and G-protein independent pathways. In the case of peptides isolated from *Rana saharica* (peaks 5.1 and 5.4), the stimulatory effects were also independent of pathways triggered by protein kinase C.

It is apparent from the insulin stimulatory effects that specific receptors must exist for these amphibian peptides on mammalian insulin-secreting beta cells. This gives rise to two major and highly novel non-exclusive possibilities. The first is that these insulin-releasing amphibian peptides have homologous or closely related mammalian counter-parts.

The second important possibility arising from this research is that the novel amphibian peptides described in Tables 1-7, or daughter molecules thereof may offer a therapeutically useful means of treating insulin secretory dysfunction and other beta cell disturbances typical of diabetes in humans. Diabetes is predicted to reach epidemic proportions throughout the world in the next 20 years and current treatments do not restore normal glucose homeostasis, therein resulting in debilitating diabetic complications and premature death. Amphibian peptides may therefore be a useful addition to the therapeutic arsenal for use either alone or in combination with other agents to improve diabetes control and decrease the risk of associated complications.

CLAIMS

1. The following peptide structures as stimulators of insulin secretion and pancreatic beta cell function:

(a) **RRKPLFPFIPRPK** (peptide 1.10, Agalychnis calcarifer)

(b) **MLADVFEKIMGD...** (N-terminus of peptide 1.7, Agalychnis litodryas)

(c) **AVWKDFLKNIGKAAGKAVLNSVTDMVNE** (peptide 2.9, Agalychnis litodryas)

(d) **Pyr-QRLGHQWAVGHLM-amidated** (peptide 21, Bombina variegata)

(e) **Pyr-DSFGNQWARGHFM-amidated** (peptide 22, Bombina variegata)

(f) **GKPFYPPPIYPEDM** (peptide 24, Bombina variegata)

(g) **IYNAICPCKHCNKCKPGLLAN** (peptide 25, Bombina variegata)

(h) **XXPLAPFFQAVFK...** (N-terminus of peptide 1.8, Phyllomedusa trinitatis)

(i) **ALWKDILKNVGKAAGKAVLNTVTDMVNQ** (peptide 2.10, Phyllomedusa trinitatis)

(j) **ALSILRGLEKLAKMGIALTNCKATKKC** (peptide 3.8, Rana palustris)

(k) **FLPIIAGVAAKVFPKIFCAISKKC** (peptide 4.1, Rana pipiens)

(l) **KGAAKGLLEVASCKLSKSC** (peptide 4.22, Rana saharica)

(m) **GIFSKFGRKKIKNLLISGLKNVGKEVGMDVVRTGIDIAGCKIKGEC** (peptide 5.1 Rana saharica)

(n) **GIFSKLAGKKLKNLLISGLKNVGKEVGMDVVRTGIDIAGCKIKGEC** (peptide 5.4 Rana saharica)

(o) **GILSTIKDFAIKAGKGAAKGLLEMASCKLSGQC** (peptide 5.6, Rana saharica)

(p) **GILLDKLKNFAKTAGKGVLQSLLNTASCKLSGQC** (peptide 6.5, Rana

saharica)

(q) **FLPLLAGLAANFLPKIFCKITRKC** (peptide 8.3, Rana saharica)

2. Brevinins, dermaseptins and esculentins as peptides that stimulate insulin secretion by activation of physiological stimulus-secretion coupling pathways rather than by antimicrobial action involving cell lysis.

3. A peptide as claimed in claims 1 and 2 with at least one amino acid modification by insertion of fatty acid at the alpha amino group of native amino acid or an epsilon amino group of a substituted lysine residue.

4. A peptide as claimed in claim 1, 2 or 3 having at least one amino acid substitution and/or modification including N-glycated,, N-alkylated, N-acetylated, N-acylated, N-isopropyl, or N-pyroglutamyl amino acids.

5. Use of a peptide as claimed in any one of claims 1 to 4 in the preparation of a medicament to stimulate insulin secretion and/or moderate blood glucose excursions.

6. The use of a peptide as claimed in any one of claims 1 to 5 in the preparation of a medicament for treatment of type 1 or type 2 diabetes mellitus.

7. A pharmaceutical composition including a peptide according to any one of claims 1 to 6.

8. A pharmaceutical composition useful in the treatment of obesity and/or type 2 diabetes which comprises an effective amount of a peptide as claimed in any of claims 1 to 7 in admixture with a pharmaceutically acceptable excipient for delivery through transdermal, nasal inhalation, oral or injected routes.

9. A pharmaceutical composition as claimed in claim 8 which further comprises one or more sulphonylureas, meglitinides, metformin, and/or thiazolidinediones.

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Table 1 Insulin secretion, experimental molecular mass, amino acid sequence, database comparison and theoretical molecular mass of individual peptides isolated from *Agalychnis calcarifer.*

Peptide ID	Insulin release (ng/10^6 cells/20mins)	Experimental Mass (Da)	Amino acid sequence	Database match	Theoretical (Calculated) Mass (Da)
None	1.71 ± 0.12				
1.3	2.52 ± 0.23**	ND	No sequence		
1.10	2.61 ± 0. 11**	1653.2	RRKPLFPFIPRPK	No Match	1652.1
1.17	2.10 ± 0.15*	ND	No sequence		
1.18	2.52 ± 0.05***	ND	No sequence		

Table 2 Insulin secretion, experimental molecular mass, amino acid sequence, database comparison and theoretical molecular mass of individual peptides isolated from *Agalychnis litodryas.*

Peptide ID	Insulin release (ng/10^6 cells/20mins)	Experimental Mass (Da)	Amino acid sequence	Database match	Theoretical (Calculated) Mass (Da)
None	1.95 ± 0.16				
1.7	4.22 ± 0.48***	2546.2	MLADVFEKIMGD ...(Insufficient sample)		
2.9	7.46 ± 0.08***	3020.0	AVWKDFLKNIGK AAGKAVLNSVTD MVNE	Dermaseptin B IV precursor 79% ID	3019.5

Incubations were performed at 5.6mM glucose. Values are mean ± SEM for 3 separate observations. **P<0.01 and ***P<0.001 compared with 5.6mM glucose. ND = Not detected. Single letter code denote amino acids: A, Ala; R, Arg; N, Asn; D, Asp; C, Cys; E, Glu; Q, Gln; G, Gly; H, His; X, Hyp; I, Ile; L, Leu; K, Lys; M, Met; F, Phe; P, Pro; S, Ser; T, Thr; W, Trp; Y, Tyr; V, Val.

Table 3 Insulin secretion, experimental molecular mass, amino acid sequence, database comparison and theoretical molecular mass of individual peptides isolated from *Bombina variegata*.

Peptide ID	Insulin release (ng/10^6 cells/20mins)	Experimental Mass (Da)	Amino acid sequence	Database match	Theoretical (Calculated) Mass (Da)
None	1.74 ± 0.08				
21	4.66 ± 0.24***	1641.7	Pyr-QRLGHQWAVG HLM-amidated	Bombesin 93% ID (His)^{6}Bombesin	1642.7
22	4.75 ± 0.13***	1662.6	Pyr-DSFGNQWARG HFM-amidated	Bombesin 72% ID	1662.9
23	5.67 ± 0.30***	1619.8	Pyr-QRLGNQWAVG HLM-amidated	Bombesin 100% ID	1620.7
24	4.30 ± 0.20***	1650.5	GKPFYPPPIYFE DM	Tryptophyllin 57% ID	1650.9
25	2.39 ± 0.30***	2300.0	IYNAICPCKHCN KCKPGLLAN	No Match	2299.8

Table 4 Insulin secretion, experimental molecular mass, amino acid sequence, database comparison and theoretical molecular mass of individual peptides isolated from *Phyllomedusa trinitatis*.

Peptide ID	Insulin release (ng/10^6 cells/20mins)	Experimental Mass (Da)	Amino acid sequence	Database Match	Theoretical (Calculated) Mass (Da)
None	1.47 ± 0.04				
1.8	2.48 ± 0.37**	8326.4	XXPLAPFFQAVFK ...(Insufficient sample)		
1.11	2.10 ± 0.16**	3379.9	ND		
2.10	2.356 ± 0.34**	2996.4	ALWKDILKNVGKA AGKAVLNTVTDMV NQ	Dermaseptin B IV precursor 100% ID	2998.5

Table 5 Insulin secretion, expetimental molecular mass, amino acid sequence, database comparison and theoretical molecular mass of individual peptides isolated from *Rana palustris*.

Peptide ID	Insulin release (ng/10^6 cells/20mins)	Experimental Mass (Da)	Amino acid sequence	Database match	Theoretical (Calculated) Mass (Da)
None	1.467 ± 0.04				
2.6	1.93 ± 0.23**	ND	ND		
2.7	4.14 ± 0.40***	8560.4	ND		
3.1	2.12 ± 0.09***	4919.9	ND		
3.8	2.48 ± 0.44**	2873.5	ALSILRGLEKLAK MGIALTNCKATKK C	Brevinin-1 (46% ID)	2873.7
4.3	2.14 ± 0.13***	3848.7	ND		
4.4	1.87 ± 0.06**	ND	ND		

Table 6 Insulin secretion, experimental molecular mass, amino acid sequence, database comparison and theoretical molecular mass of individual peptides isolated from *Rana pipiens*.

Peptide ID	Insulin release (ng/10^6 cells/20mins)	Experimental Mass (Da)	Amino acid sequence	Database match	Theoretical (Calculated) Mass (Da)
None	2.76 ± 0.13				
3.1	3.46 ± 0.17***	5125.2	ND		
4.1	4.15 ± 0.01***	2562.6	FLPIIAGVAAKV FPKIFCAISKKC	Pipinin-1 100% ID)	2563.2

Incubations were performed at 5.6mM glucose. Values are mean ± SEM for 3 separate observations. **P<0.01 and ***P<0.001 compared with 5.6mM glucose. ND = Not detected. Single letter code denote amino acids: A, Ala; R, Arg; N, Asn; D, Asp; C, Cys; E, Glu; Q, Gln; G, Gly; H, His; X, Hyp; I, Ile; L, Leu; K, Lys; M, Met; F, Phe; P, Pro; S, Ser; T, Thr; W, Trp; Y, Tyr; V, Val

Table 7 Insulin secretion, experimental molecular mass, amino acid sequence, database comparison and theoretical molecular mass of individual peptides isolated from *Rana saharica*.

Peptide ID	Insulin release (ng/10^6 cells/20mins)	Experimental Mass (Da)	Amino acid sequence	Database match	Theoretical (Calculated) Mass (Da)
None	1.87 ± 0.06				
4.14	3.15 ± 0.23**	ND	ND		
4.18	3.52 ± 0.21***	ND	ND		
4.22	3.47 ± 0.40***	1892.6	KGAAKGLLEVASC KLSKSC	Rugosin A 68.4% ID	1891.2
4.23	4.25 ± 0.17***	2930.8	AVITGACERDVQC GGGTCCAVSLI.... (insufficient sample)	Protein A/BV8 78% ID	2322.6
4.26	3.08 ± 0.19**	1433.7	ND		
4.27	3.09 ± 0.23**	ND	ND		
4.28	3.19 ± 0.08***	ND	ND		
5.1	3.32 ± 0.22***	4920.4	GIFSKFGRKKIKNL LISGLKNVGKEVG MDVVRTGIDIAGC KIKGEC	Esculentin -1 98% ID	4919.2
5.2	2.95 ± 0.08***	3404.6	ND		
5.3	2.54 ± 0.15**	ND	ND		
5.4	3.30 ± 0.22***	4801.2	GIFSKLAGKKLKN LLISGLKNVGKEV GMDVVRTGIDIAG CKIKGEC	Esculentin -1B 100% ID	4800.8
5.6	2.86 ± 0.37**	3309.2	GILSTIKDFAIKAG KGAAKGLLEMASC KLSGQC	Brevinin-2EB 67%ID	3309.0
6.5	5.93 ± 0.47***	3519.3	GILLDKLKNFAKT AGKGVLQSLLNTA SCKLSGQC	Brevinin-2EC 100% ID	3519.2
6.7	3.46 ± 0.28**	3119.2	ND		
8.3	3.53 ± 0.06***	2676.9	FLPLLAGLAANFLP KIFCKITRKC	Brevinin-1E 100% ID	2676.4

Incubations were performed at 5.6mM glucose. Values are mean ± SEM for 3 separate observations. **P<0.01 and ***P<0.001 compared with 5.6mM glucose. ND = Not detected. Single letter code denote amino acids: A, Ala; R, Arg; N, Asn; D, Asp; C, Cys; E, Glu; Q, Gln; G, Gly; H, His; X, Hyp; I, Ile; L, Leu; K, Lys; M, Met; F, Phe; P, Pro; S, Ser; T, Thr; W, Trp; Y, Tyr; V, Val

Table 8 Effects of the 13 amino acid peptide peak (peak 1.10) from ***Agalychnis calcarifer*** on iInsulin secretion from BRIN-BD11 cells in the presence of verapamil or a depolarising K^+ concentration.

Addition	Insulin secretion (ng/10^6 cells /20 min)	
	Control	Peak 1.10
None	1.68 ± 0.16	2.75 ± 0.12 *
Verapamil (50μM)	1.74 + 0.17	3.54 ± 0.20 $^{\Delta\Delta}_{***}$
KCl (30mM)	5.32 + 0.38 $^{\Delta\Delta\Delta}$	16.45 ± 0.10 $^{\Delta\Delta}_{***}$

Table 9 Effects of peaks 21, 22, 23 and 24 from ***Bombina variegata*** on iInsulin secretion from BRIN-BD11 cells in the presence of verapamil or a depolarising K^+ concentration.

Addition	Insulin secretion (ng/10^6 cells /20 min)				
	Control	Peak 21	Peak 22	Peak 23	Peak 24
None	1.69 ± 0.17	3.65 ± 0.05***	3.23 ± 0.14***	3.40 ± 0.14***	3.31 ± 0.20***
Verapamil (50μM)	1.74 ± 0.17	4.25 ± 0.82***	3.05 ± 0.09***	3.21 ± 0.14***	3.08 ± 0.29***
KCl (30mM)	5.33 ± 0.38 $^{\Delta\Delta\Delta}$	13.68 ± 1.42 $^{\Delta\Delta\Delta}_{***}$	13.69 ± 1.43 $^{\Delta\Delta\Delta}_{***}$	15.21 ± 1.43 $^{\Delta\Delta\Delta}_{***}$	15.35 ± 0.41 $^{\Delta\Delta\Delta}_{***}$

Acute incubations were performed at 5.6 mM glucose. Values are mean ± SEM for 8 separate observations. *P<0.05 and ***P<0.001 compared with control, $^{\Delta\Delta}$P<0.01 and $^{\Delta\Delta\Delta}$P<0.001 compared with no addition.

Table 10 Effects of peaks 5.1 and 5.4 from *Rana saharica* on iInsulin secretion from BRIN-BD11 cells in the presence of verapamil or a depolarising K^+ concentration.

Addition	Insulin secretion (ng/10^n cells/20 min)		
	Control	Peak 5.1	Peak 5.4
None	1.69 ± 0.17	2.96 ± 0.32***	2.89 ± 0.19***
Verapamil (50μM)	1.74 ± 0.17	2.88 ± 0.28***	2.79 ± 0.10***
KCl (30mM)	5.33 ± 0.38ΔΔΔ	10.11 ± 0.81 ΔΔΔ ***	11.84 ± 0.98 ΔΔΔ ***

Acute incubations were performed at 5.6 mM glucose. Values are mean ± SEM for 8 separate observations. ***P<0.001 compared with control, ΔΔΔP<0.001 compared with no addition.

Legends to Figures

Figure 1 Effects of various semi-preparative C18 HPLC fractions of *Agalychnis calcarifer* crude venom on insulin secretion from BRIN-BD11 cells. Incubations were performed at 5.6mM glucose. Values are the mean ± SEM for 3 separate observations. *P<0.01and **P<0.001 compared with 5.6mM glucose alone.

Figure 2 Effects of various semi-preparative C18 HPLC fractions of *Agalychnis litodryas* crude venom on insulin secretion from BRIN-BD11 cells. Incubations were performed at 5.6mM glucose. Values are the mean ± SEM for 3 separate observations. *P<0.01and **P<0.001 compared with 5.6mM glucose alone.

Figure 3 Effects of various semi-preparative C18 HPLC fractions of *Bombina variegata* crude venom on insulin secretion from BRIN-BD11 cells. Incubations were

performed at 5.6mM glucose. Values are the mean ± SEM for 3 separate observations. *P<0.01and **P<0.001 compared with 5.6mM glucose alone.

Figure 4 Effects of various semi-preparative C18 HPLC fractions of ***Phyllomedusa trinitatis*** crude venom on insulin secretion from BRIN-BD11 cells. Incubations were performed at 5.6mM glucose. Values are the mean ± SEM for 3 separate observations. *P<0.001and **P<0.001 compared with 5.6mM glucose alone.

Figure 5 Effects of various semi-preparative C18 HPLC fractions of ***Rana palustris*** crude venom on insulin secretion from BRIN-BD11 cells. Incubations were performed at 5.6mM glucose. Values are the mean ± SEM for 3 separate observations. *P<0.01and **P<0.001 compared with 5.6mM glucose alone.

Figure 6 Effects of various semi-preparative C18 HPLC fractions of *Rana pipiens* crude venom on insulin secretion from BRIN-BD11 cells. Incubations were performed at 5.6mM glucose. Values are the mean ± SEM for 3 separate observations. *P<0.01and **P<0.001 compared with 5.6mM glucose alone.

Figure 7 Effects of various semi-preparative C18 HPLC fractions of *Rana saharica* crude venom on insulin secretion from BRIN-BD11 cells. Incubations were performed at 5.6mM glucose. Values are the mean ± SEM for 3 separate observations. **P<0.001 compared with 5.6mM glucose alone.

Figure 8 Acute effects of the 13 amino acid 1653.2 Da purified peptide (peak 1.10) from *Agalychnis calcarifer* on forskolin and PMA on insulin secretion from BRIN-BD11 cells cultured overnight in the absence (control) and presence of 25 μM forskolin, 10 nM PMA or 0.1μg/ml pertussis toxin. Acute incubations were performed

at 5.6 mM glucose. Values are the mean ± SEM for 8 separate observations. ***P<0.001 compared with 5.6 mM glucose alone under same culture conditions. $^{\Delta\Delta\Delta}$P< 0.001 compared with respective test reagent following control culture.

Figure 9 Acute effects of 1641.7, 1662.6, 1619.8 and 1650.5 Da purified peptides (peaks 21, 22, 23 and 24) from *Bombina variegata* on forskolin and PMA on insulin secretion from BRIN-BD11 cells cultured overnight in the absence (control) and presence of 25 µM forskolin, 10 nM PMA or 0.1µg/ml pertussis toxin. Acute incubations were performed at 5.6 mM glucose. Values are the mean ± SEM for 8 separate observations. **P<0.001 compared with 5.6 mM glucose alone under same culture conditions. $^{\Delta}$P< 0.001 compared with respective test reagent following control culture.

Figure 10 Acute effects of 4920.4 and 4801.2 Da purified peptides (peaks 5.1 and 5.4) from *Rana saharica* on forskolin and PMA on insulin secretion from BRIN-BD11 cells cultured overnight in the absence (control) and presence of 25 µM forskolin, 10 nM PMA or 0.1µg/ml pertussis toxin. Acute incubations were performed at 5.6 mM glucose. Values are the mean ± SEM for 8 separate observations. ***P<0.001 compared with 5.6 mM glucose alone under same culture conditions. $^{\Delta\Delta}$P< 0.001 compared with respective test reagent following control culture.

Figure 1 illustrates that semi-preparative C18 HPLC fractions of the skin secretions of *Agalychnis calcarifer* stimulate insulin secretion from BRIN-BD11 cells.

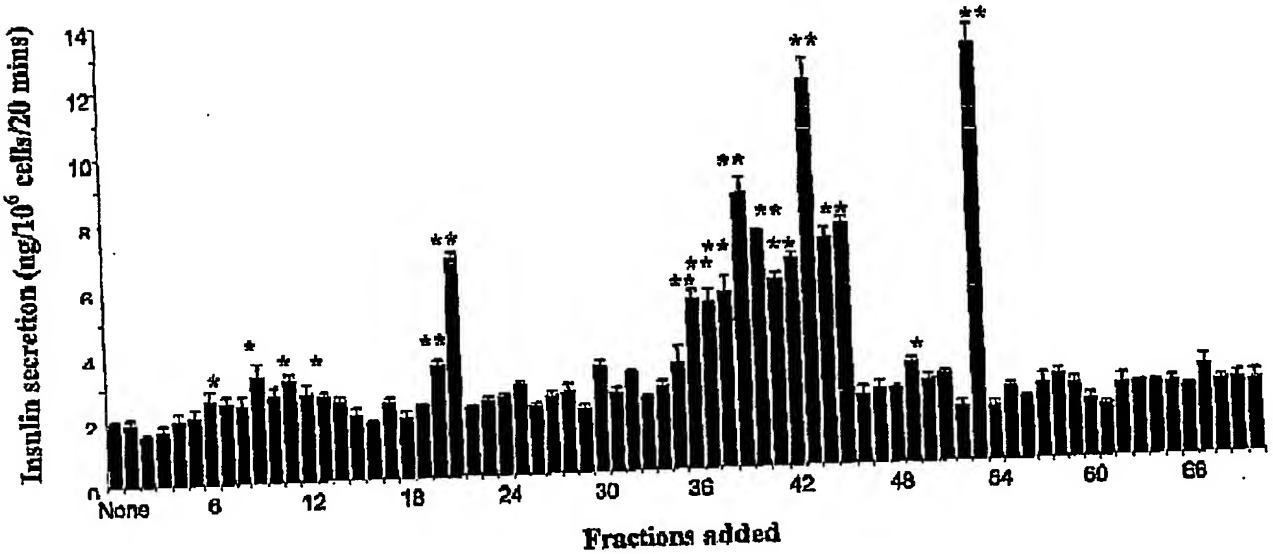

Figure 2 illustrates that semi-preparative C18 HPLC fractions of the skin secretions of *Agalychnis litodryas* stimulate insulin secretion from BRIN-BD11 cells.

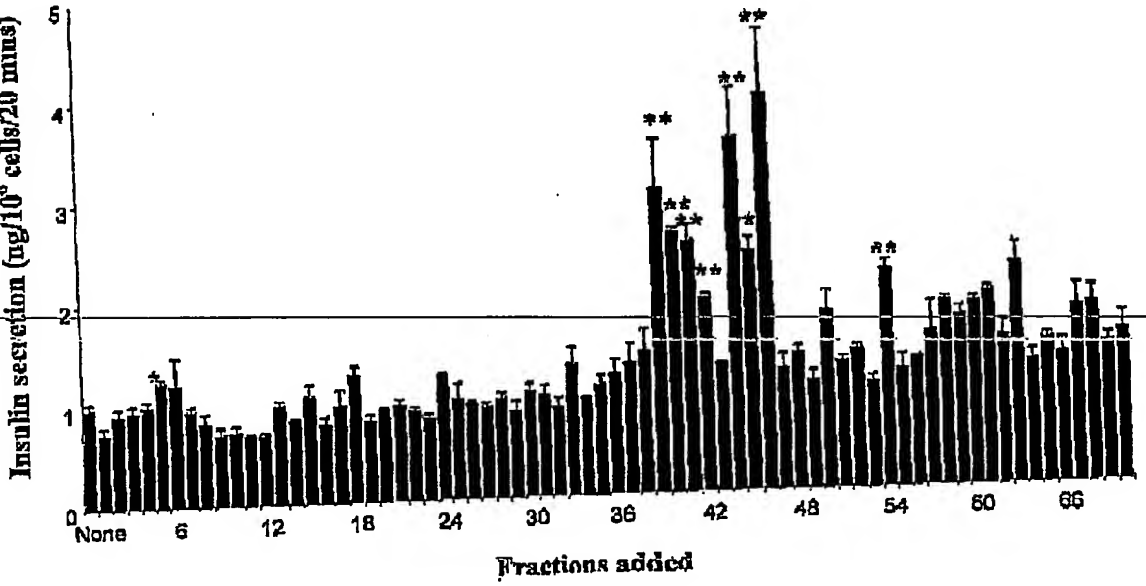

Figure 3 illustrates that semi-preparative C18 HPLC fractions of the skin secretions of *Bombina variegata* stimulate insulin secretion from BRIN-BD11 cells.

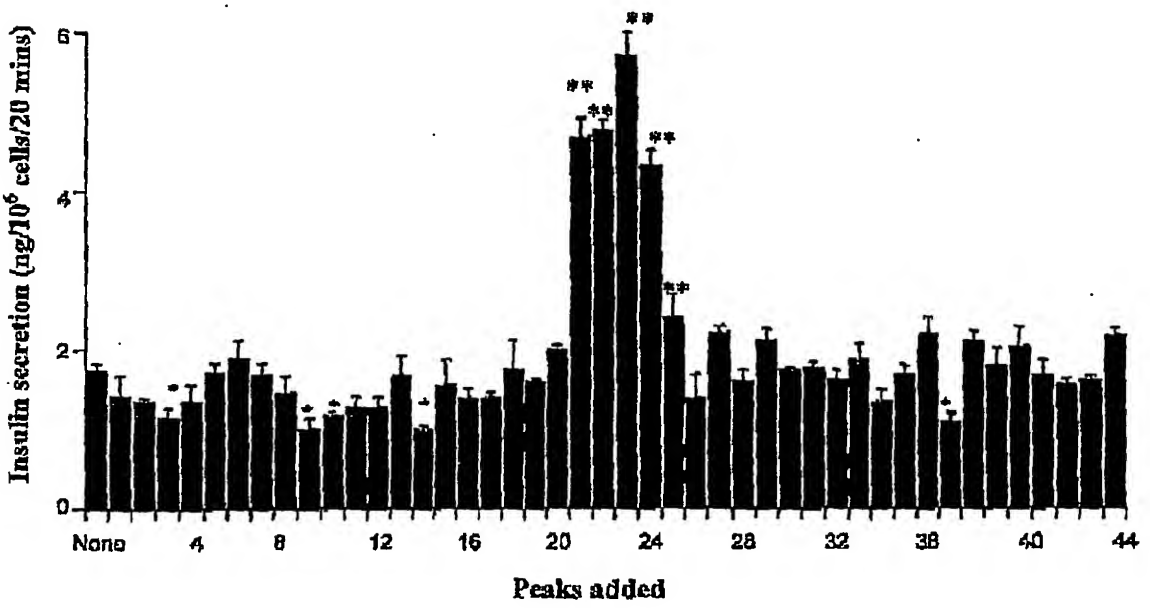

Figure 4 illustrates that semi-preparative C18 HPLC fractions of the skin secretions of *Phyllomedusa trinitatis* stimulate insulin secretion from BRIN-BD11 cells.

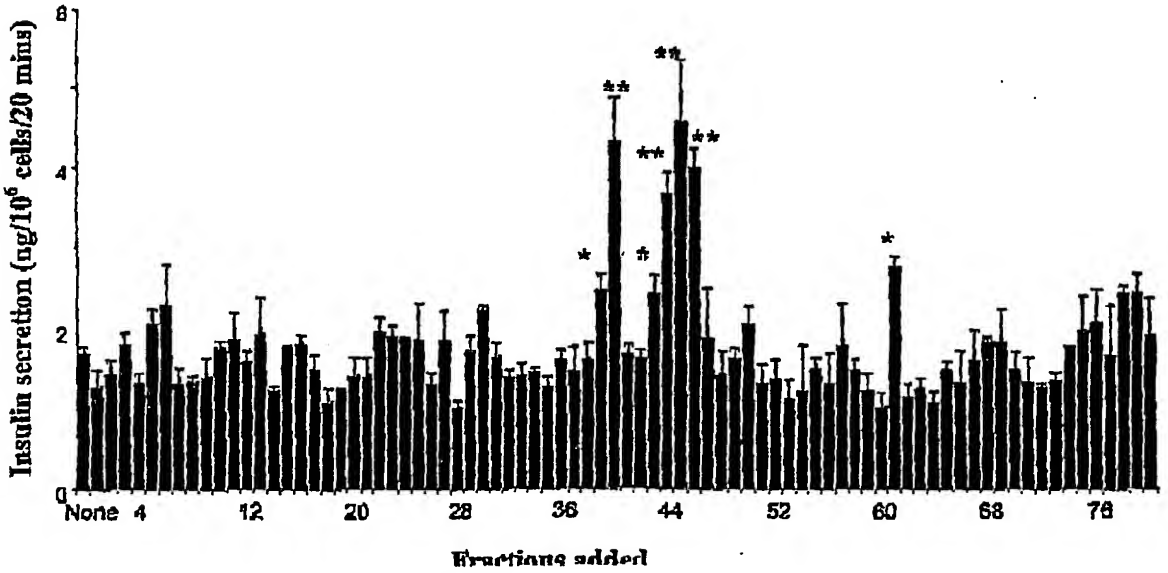

Figure 5 illustrates that semi-preparative C18 HPLC fractions of the skin secretions of *Rana palustris* stimulate insulin secretion from BRIN-BD11 cells.

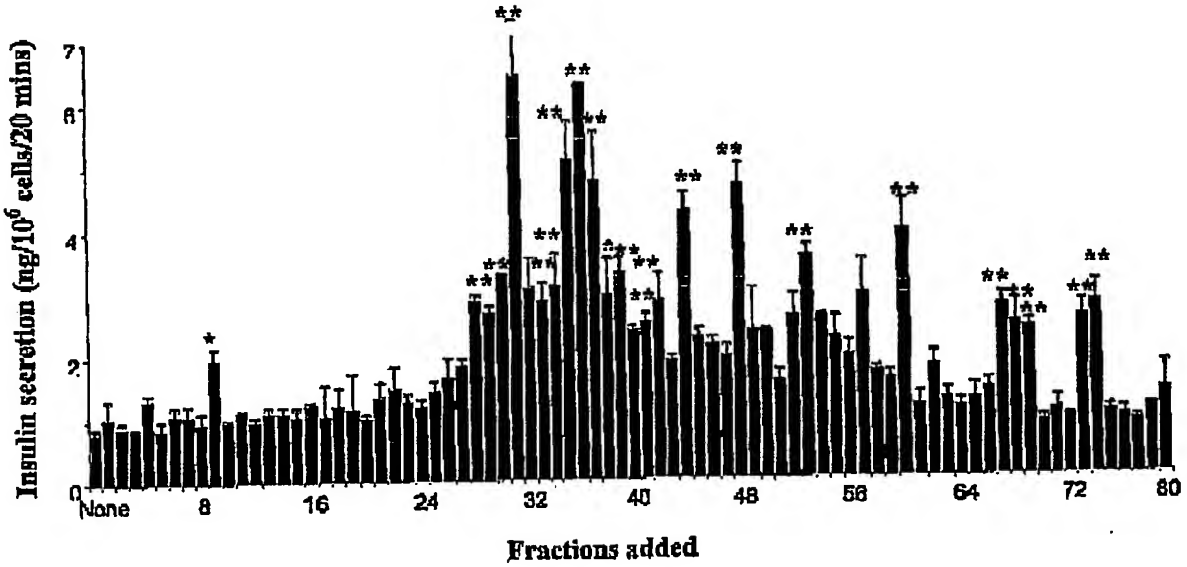

Figure 6 illustrates that semi-preparative C18 HPLC fractions of the skin secretions of *Rana pipiens* stimulate insulin secretion from BRIN-BD11 cells.

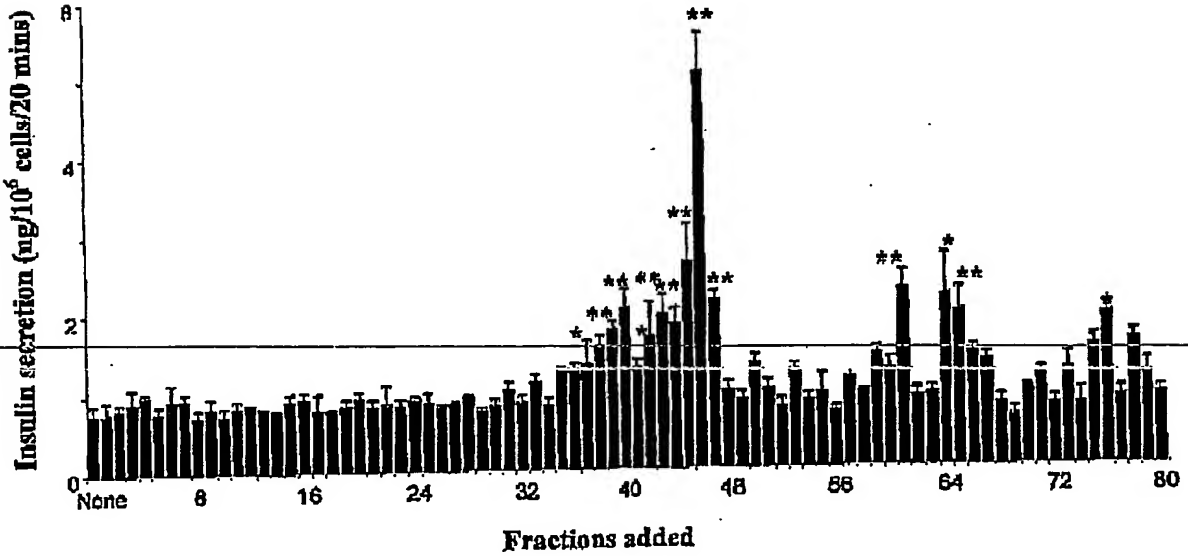

Figure 7 illustrates that semi-preparative C18 HPLC fractions of the skin secretions of *Rana saharica* stimulate insulin secretion from BRIN-BD11 cells.

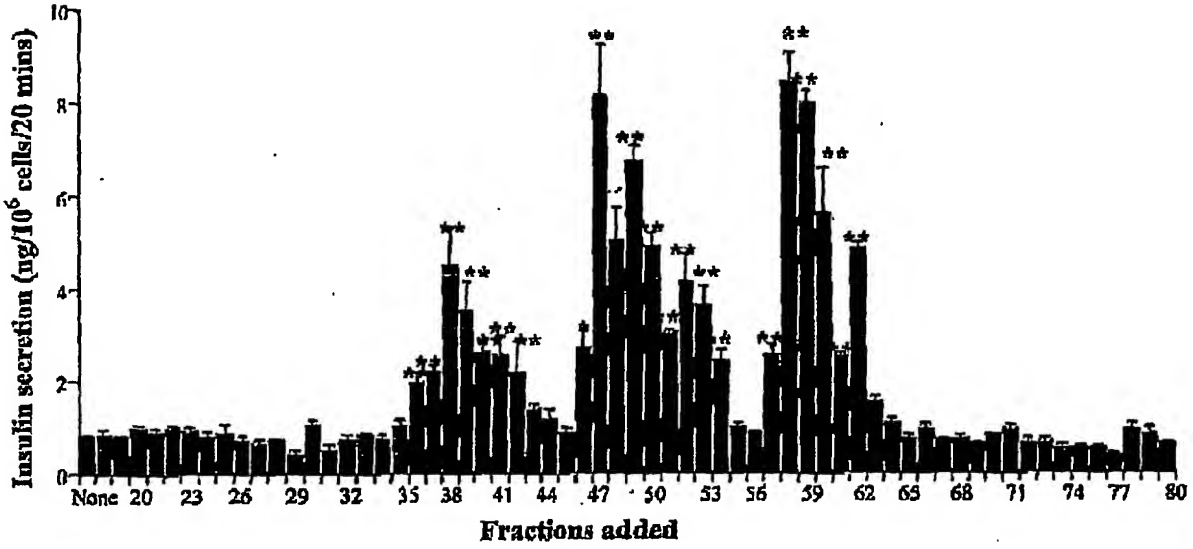

Figure 8 illustrates the dependence of the stimulatory effects of 1653.2 Da purified peptide (peak 1.10) from *Agalychnis calcarifer* on intracellular pathways mediated by protein kinase A (forskolin), protein kinase C (PMA) or pertussis toxin-sensitive G-proteins.

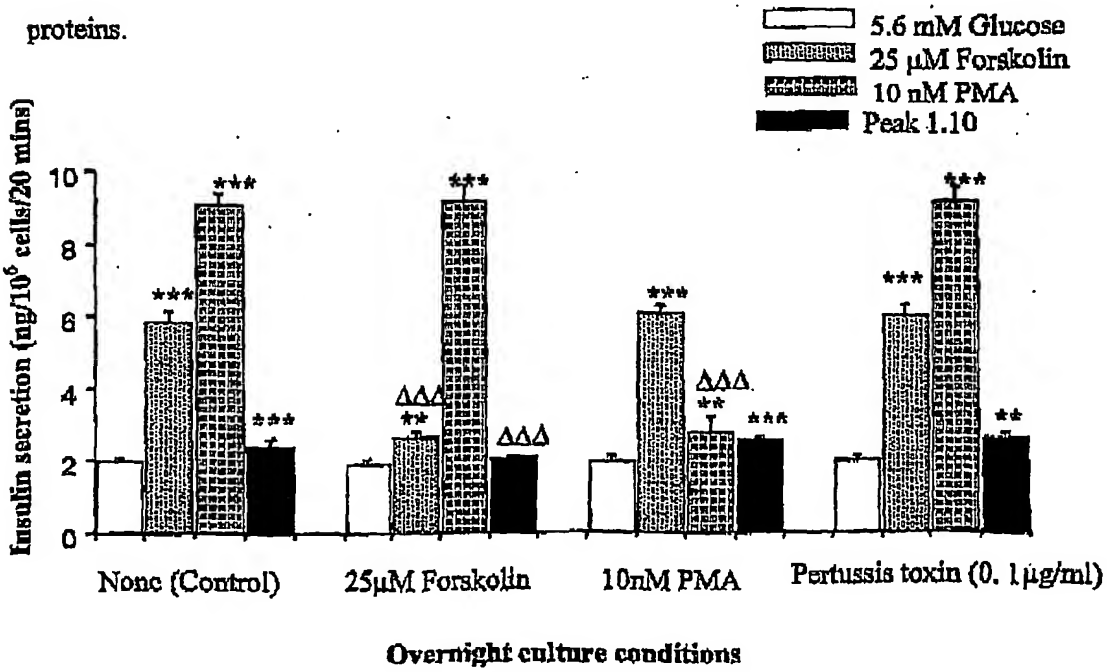

Figure 9 illustrates the dependence of the stimulatory effects of 1641.7 Da, 1662.6 Da, 1619.8 Da and 1650.5 Da purified peptides (peaks 21, 22, 23 and 24) from *Bombina variegata* on intracellular pathways mediated by protein kinase A (forskolin), protein kinase C (PMA) or pertussis toxin-sensitive G-proteins.

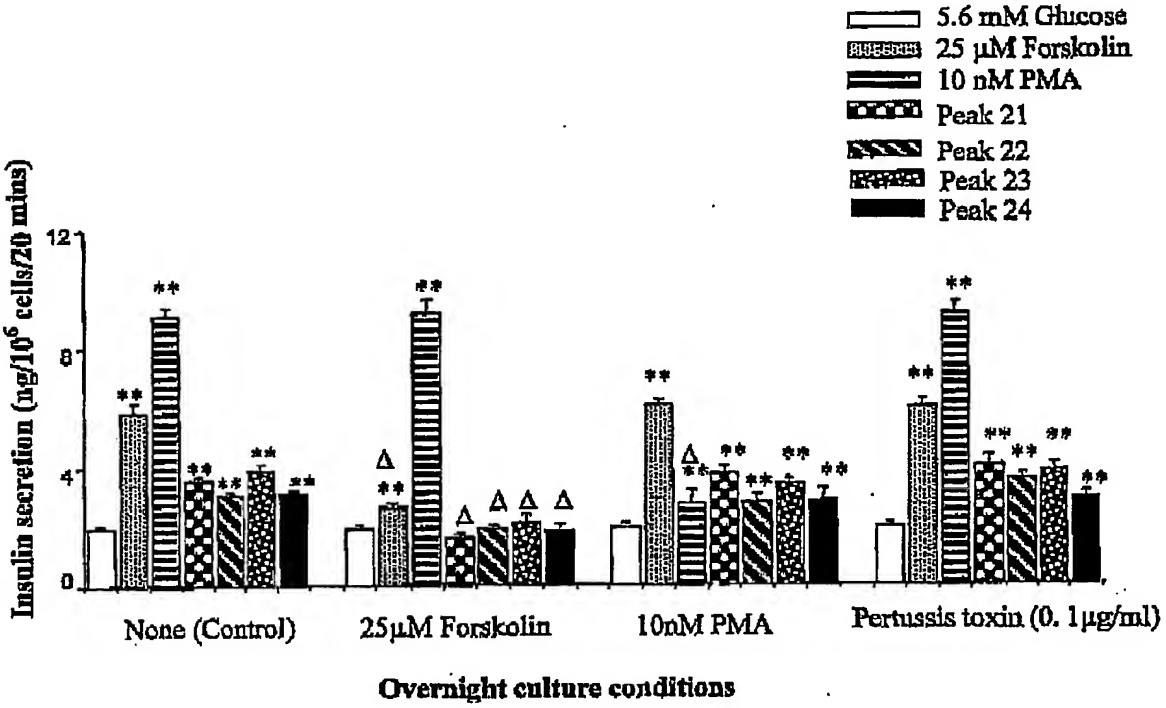

Figure 10 illustrates the dependence of the stimulatory effects of 4920.4 Da and 4801.2 Da purified peptides (peaks 5.1 and 5.4) from *Rana saharica* on intracellular pathways mediated by protein kinase A (forskolin), protein kinase C (PMA) or pertussis toxin-sensitive G-proteins.